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## *FINAL REPORT*

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# DEVELOPMENT OF N2-FIXING AND CELLUOSE DEGRADING MICROBIAL INOCULANT TO CONVERT STRAW RESIDUE INTO A N-ENRICHED ORGANIC FERTILIZER

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# Development of $N_2$-fixing and cellulose degrading microbial inoculant to convert straw residue into a N-enriched organic fertilizer

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**Abstract:**

The objective of this research was to develop a microbial inoculant to enrich N content of organic residues. A number of $N_2$ fixing bacteria and cellulose degrading bacteria were isolated from soils and straw residues from organic farms. These bacteria were identified by GC-FAME analysis and assessed for their ability to act synergistically to degrade straw residue and increase residue N content through $N_2$ fixation. The relative $N_2$ fixation potential of 33 bacteria isolates and the relative cellulose degradation potential of 65 bacterial isolates were determined. A majority of the $N_2$ fixing bacteria were species belonging to the genera *Bacillus* or *Paenibacillus*. The most active identified cellulose degraders were species in the genera *Paenibacillus*, *Bacillus*, *Curtobacterium* and *Sphingobacterium*. Experiments with combinations of cellulose degrading and $N_2$ fixing bacteria indicated that from 4% up to 17% of added straw residue was degraded, and in some instance $N_2$ fixation was supported. In these incubation studies, the degraded residue contained increased amounts of N relative to the original starting residue. Microcosm compost studies suggest that application of mixed inoculants for producing large amounts of N enriched straw residues is limited.

**Technical Report:**

**Introduction**: Organic production systems represent an economically significant niche in Saskatchewan agriculture. These systems require intensive management of essential soil nutrients to be a sustainable system that maintains soil fertility status. Inorganic fertilizers provide Nitrogen (N), a key nutrient in conventional production systems. These fertilizers however, are not allowed in organic production systems. Rather, green manure using $N_2$ fixing legumes allow organic producers to increase N status of their soil. The objective of this study is to develop an alternative method for organic producers to increase the level of soil N. This study will utilize free-living $N_2$ fixing and cellulose-degrading microorganisms to produce an inoculant to enrich the N content of straw and other low N organic residues (Halsall, 1993). The $N_2$ fixing and cellulose degrading microorganisms will be isolated from soils collected from organic farms in Saskatchewan. The most efficient organisms will be developed into an inoculant where the cellulose degrading microorganisms break down the resistant components of the straw residues (i.e., cellulose) into more readily used organic substrates so that the free living $N_2$ fixing bacteria can convert atmospheric nitrogen into organic nitrogen (Halsall, 1993, Magan et al 1988, and Halsall and Gibson, 1985). This biological inoculant may then be used by organic producers on farm sites to help manage and recycle organic residues and to create a N-enriched fertilizer.

**Research Objectives**:

The long-term objective of this research was to develop an inoculant to enrich N content of organic residues.

The short-term objectives of this research were:

Year 1) To isolate and identify free living $N_2$ fixing bacteria and cellulose degrading microorganisms found in soils from organic farms in Saskatchewan.

Year 2) To characterize the ability of isolates to fix N or degrade cellulose and to select the best inoculant strains.

Year 3) To assess various combinations of $N_2$ fixing and cellulose degrading bacteria as inoculants to degrade and enrich N content of various residues in growth chamber and small plot studies.

**A) Description of work completed:**
**Progress on all objectives has been reported in previous reports. Here, key findings and final conclusions are provided.**

**Nitrogen fixing bacteria:** Over 300 bacterial isolates were screened for their ability to fix $N_2$. A total of 33 isolates were confirmed positive for $N_2$ fixation activity (i.e., acetylene reduction assay - ARA). Of these isolates, 24 were identified by GC MIDI FAME analysis and eight were not found in the database. Not surprising, a majority of the $N_2$ fixing bacteria were species belonging to the genera *Bacillus* or *Paenibacillus*. The relative $N_2$ fixation potential of these isolates is given in Table 1. It was observed that nine of these isolates were actually a consortium of two bacteria – some of which lack $N_2$ fixation potential when cultured individually. A total of 14 isolates exhibited ARA between 15% to over 450% greater than that of the positive control bacterium, *Bacillus polymyxa*. These results show that free-living, asymbiotic $N_2$ fixing bacteria were readily isolated from soil and crop residues. A culture collection of these $N_2$ bacteria was established and now available for future research.

**Cellulose degrading bacteria**: Sixty-five cellulose-degrading bacterial isolates were assessed using a filter paper degradation assay. The relative ability of these isolate to degrade cellulose is based on their ability to release reducing sugars from cellulose. Cellulose degrading activity of isolates varied significantly and the most active degraders are listed in Table 2. The most active identified cellulose degrader were species in the genera *Paenibacillus*, *Bacillus*, *Curtobacterium* and *Sphingobacterium*. Almost 50% of the cellulose degrades could not be identified using the GC MIDI FAME system. These results indicated that a variety of cellulose degrading bacteria colonize crop residues and could be readily isolated. A culture collection of these cellulose-degrading bacteria was established and now available for future research.

**Studies on composting straw with cellulose degrading and $N_2$ fixing bacteria:** Twenty-eight combinations of cellulose degrading and $N_2$ fixing bacteria were tested as mixed cultures for their ability to degrade wheat straw, and increase nitrogen content of residue. The best combination degraded between 3% and 17.6% of the initial straw residue. Interestingly, the best combination consisted of the cellulose degrading isolate *Bacillus subtilis* SP2 – C20 and the $N_2$ fixing isolate *Paenibacillus polymyxa* SP2 –N3, neither of which was the most active in its respective ability. Based on the ARA test, it seems that some levels of nitrogen fixation were detected in decomposing wheat straw residues, whereas less ARA was detected in decomposing pea crop residues. The carbon and nitrogen analysis on the decomposed wheat straw residue suggest that some inoculants increased the relative amount of N in the decomposed residue relative to the original material (Table 3). This small-scale incubation study shows that it might be possible to use mixed inoculants to degrade straw and increase the N content of the resulting residues. In contrast, in some inoculants produced extracellular polysaccharides during decomposition of the straw.

**Compost Study:** A microcosm study was conducted to assess three combinations of cellulose degrading bacteria and nitrogen fixing bacteria for their ability to degrade larger quantities of wheat straw. The mixed cultures tested were: the cellulose degrading isolates *Bacillus subtilis* SP2 – C20 and the $N_2$ fixing isolates *Paenibacillus polymyxa* SP2 –N3; *Paenibacillus macerans* MS2–C8 and *Paenibacillus azotofixans* CG2–N7b, and, *Paenibacillus gordonae* GW1–C36 and *Paenibacillus polymyxa* CP1–N1. These cultures were selected to provide a range of cellulose degrading and $N_2$ fixation (ARA) activity. Cultures were grown as for initial experiments and used to inoculate 50 – 100 grams of wheat straw in mini-compost microcosms. The microcosms were incubated at 22°C and routinely mixed and aerated, watered. The mini-compost microcosms did not work as well as expected and the wheat straw decomposed every slowly relative to initial experiments. During four months of incubation samples were assessed for ARA activity at monthly intervals. Little if any ARA activity was detected in the microcosms. At the end of the composting period the change in the % N in residue was relatively small at less than 1%. The study showed that use of mixed bacterial cultures to enhance N content of composting residues was more complicated than in lab assays.

**Key finding:** Mixed bacterial cultures hold potential as inoculants to convert crop residues into organic fertilizers, but large-scale application of bacterial inoculants could be complicated and fail to yield desired

results. Future research needs to assess factors affecting success of composting systems that might be used to produce N-enriched residues.

**Deviations from Original Work Plan**: No major deviations.; although $^{15}N$ studies were not conducted due to expense and time.

**Achievements**: No publications or conference presentations to report at this time.

**NB**: **CARC–IARC** information:
http://res1.agr.ca/pls/icarwcb/icarqueryeng.display?p_year=2002&p_icar_id=33330851

**Equipment**: None purchased.

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**Table 1. Relative $N_2$ fixation potential (ARA) of bacterial cultures**

| Isolate | Identification | Sim Index | Relative Acetylene Reduction Activity (ARA) |
|---|---|---|---|
| BN2-N7b | *Agrobacterium radiobacter* | 0.854 | 4474 |
| CG2-N11a | *Burkholderia cepacia* | 0.575 | 2590 |
| BN2-N17b | *Bacillus cereus* | 0.722 | 2457 |
| KP2-N136b | *Unknown* | | 1700 |
| OX1-N107b | *Unknown* | | 1670 |
| KP2-N136a | *Paenibacillus macerans* | 0.197 | 1649 |
| QA1-N61a | *Paenibacillus polymyxa* | 0.785 | 1619 |
| YK1-N18a | *Alcaligenes piechaudii / Comamonas testosteroni* | 0.371 / 0.266 | 1558 |
| BN2-N27c | *Paenibacillus polymyxa / unknown* | 0.953 | 1402 |
| SP2-N3 | *Paenibacillus polymyxa* | 0.897 | 1390 |
| HB3-N5 | *Xanthobacter agilis / Pseudomonas putida* | 0.356 / 0.213 | 1376 |
| CG2-N7b | *Paenibacillus azotofixans / unknown* | 0.334 | 1168 |
| CG2-N20a | *Paenibacillus macerans / Bacillus circulans* | 0.118 / 0.180 | 1084 |
| Positive control | *Bacillus polymyxa* | 0.769 | 937 |
| BN2-N18a | *Paenibacillus polymyxa / Paenibacillus polymyxa* | 0.658 / 0.718 | 897 |
| MS2-N44a | *Unknown* | | 633 |
| KP2-N167a | *Unknown* | | 556 |
| CP1-N1 | *Sphingobacterium spiritovorum / Paenibacillus polymyxa* | 0.825 / 0.877 | 524 |
| BN2-N30a | *Paenibacillus polymyxa / Paenibacillus polymyxa* | 0.841 / 0.725 | 456 |
| QA1-N61c | *Unknown* | | 374 |
| CP1-N9 | *Paenibacillus polymyxa / Paenibacillus polymyxa* | 0.974 / 0.615 | 313 |
| BN2-N9 | *Paenibacillus polymyxa / unknown* | 0.835 | 301 |
| BN2-N18b | *Bacillus megaterium* | 0.662 | 299 |
| KP2-N130a | *Unknown* | | 294 |
| KP2-N122a | *Paenibacillus polymyxa* | 0.859 | 242 |
| BN2-N27a | *Paenibacillus polymyxa* | 0.965 | 203 |
| BN2-N29 | *Paenibacillus polymyxa* | 0.804 | 87 |
| BN2-N26a | *Unknown* | | 63 |
| QA1-N61b | *Unknown* | | 63 |
| BN2-N17a | *Paenibacillus polymyxa* | 0.743 | 40 |
| CG2-N35 | *Bacillus circulans* | 0.310 | 37 |
| BN2-N25b | *Paenibacillus polymyxa / Paenibacillus pabuli* | 0.865 / 0.501 | 37 |
| BN2-N14 | *Paenibacillus polymyxa / Paenibacillus polymyxa* | 0.699 / 0.924 | 29 |
| GW1-N33 | *Paenibacillus polymyxa* | 0.717 | 28 |

Note: Several hundred colonies were selected and tested for acetylene reduction. Some cultures exhibiting nitrogen fixation were a combination of two or more bacteria. Cultures were purified and identified by FAME, but not all of these purified cultures exhibited nitrogen fixation activity on their own.

**Table 2: Relative degradation of cellulose by selected bacterial isolates as determined by production of reducing sugars**

| Isolate | Identification | Sim Index | Concentration of reducing sugar (mg/ml) |
|---|---|---|---|
| GW1-C36 | *Paenibacillus gordonae* | 0.638 | 2.855 |
| RL1-C37 | *Unknown* | | 2.828 |
| KP1-C31 | *Unknown* | | 2.748 |
| GW1-C58 | *Bacillus pumilus* | 0.653 | 2.673 |
| KP1-C2 | *Pseudomonas putida* | 0.393 | 2.673 |
| KP1-C13b | *Unknown* | | 2.472 |
| GW1-C26 | *Unknown* | | 2.381 |
| GW1-C39 | *Unknown* | | 1.966 |
| OX1-C47 | *Unknown* | | 1.676 |
| CP1-C17b | *Unknown* | | 1.561 |
| KP1-C12 | *Curtobacterium flaccumfaciens* | 0.521 | 1.511 |
| MS2-C14 | *Sphingobacterium multivorum* | 0.630 | 1.186 |
| SN2-C20 | *Bacillus subtilis* | 0.492 | 1.183 |
| MS2-C23 | *Paenibacillus peoriae* | 0.288 | 1.106 |
| MS2-C92b | *Bacillus pumilus* | 0.831 | 1.090 |
| KP1-C13a | *Unknown* | | 1.090 |
| CP1-C59 | *Unknown* | | 1.054 |
| MS2-C77 | *Bacillus pumilus* | 0.767 | 1.051 |
| MS2-C95 | *Paenibacillus pabuli* | 0.803 | 1.048 |
| MS2-C3 | *Sphingobacterium multivorum* | 0.834 | 1.013 |
| CP1-C31 | *Unknown* | | 0.963 |
| CP1-C90 | *Arthrobacter oxydans* | 0.690 | 0.941 |
| RL1-C64 | *Unknown* | | 0.938 |
| OX1-C49b | *Unknown* | | 0.935 |
| CP1-C5 | *No match* | | 0.924 |
| CP1-C41 | *No match* | | 0.922 |
| SN2-C54 | *Bacillus lichenformis* | 0.622 | 0.908 |
| KP1-C32 | *Unknown* | | 0.908 |
| RC1-C67 | *Unknown* | | 0.902 |
| MS2-C8 | *Paenibacillus macerans* | 0.481 | 0.880 |
| OX1-C15 | *Cellulomonas fimi* | 0.104 | 0.880 |
| RT2-C3b | *Curtobacterium citreum* | 0.723 | 0.870 |
| CP1-C43a | *No match* | | 0.862 |
| MS2-C18 | *Sphingobacterium multivorum* | 0.626 | 0.853 |
| RT2-C24a | *Paenibacillus polymyxa* | 0.678 | 0.834 |
| CP1-C13 | *No match* | | 0.824 |
| RC1-C68 | *Curtobacterium flaccumfaciens* | 0.364 | 0.823 |
| MS2-C92a | *Bacillus pumilus* | 0.689 | 0.820 |
| CP1-C105 | *No match* | | 0.820 |
| MS2-C8 | *Paenibacillus macerans* | 0.481 | 0.809 |
| Blank | | | 0.066 |

Note: Concentration of reducing sugar for remaining 26 isolates tested was below 0.800 mg/ml. Calculations based on reference to glucose standards graph.

**Table 3: Decomposition of different crop residues by microbial inoculants, and relative changes in the % C and N in residue**

| Inoculants | Residue Used | % Decomposition | N2 fixation (ARA) | % C in residue | % Change in C content of residue | % N in residue | % Change in N content of residue |
|---|---|---|---|---|---|---|---|
| SP2-N3 and SN2-C20 | Spring wheat (SN2) | 14.00 | 6.253 | 33.90 | - 1.35 | 1.25 | + 24.01 |
| SP2-N3 and SN2-C54 | Spring wheat (SN2) | 13.00 | 1.093 | 30.87 | - 10.17 | 1.24 | + 22.72 |
| BN2-N7b and MS2-C77 | Spring wheat (MS2) | 6.00 | 2.923 | 25.80 | - 24.92 | 1.17 | + 16.37 |
| CG2-N7b and MS2-C8 | Spring wheat (MS2) | 13.50 | 13.036 | 29.80 | - 13.28 | 1.05 | + 4.17 |
| BN2-N17b and SN2-C20 | Spring wheat (SN2) | 11.00 | 1.649 | 34.33 | - 0.10 | 0.89 | - 11.51 |
| YK1-N18a and MS2-C77 | Spring wheat (MS2) | 7.00 | 7.772 | 32.40 | - 5.71 | 0.98 | - 3.08 |
| BN2-N17b and SN2-C54 | Spring wheat (SN2) | 9.00 | 3.208 | 34.83 | + 1.36 | 0.94 | - 6.75 |
| BN2-N27c and SN2-C54 | Spring wheat (SN2) | 10.50 | 0 | 33.87 | - 1.43 | 1.00 | 0.79 |
| CP1-N1 and GW1-C36 | Spring wheat (GW1) | 10.50 | 9.306 | 43.10 | + 25.43 | 0.93 | - 7.84 |
| BN2-N17b and SN2-C31 | Spring wheat (SN2) | 10.00 | 3.678 | 30.80 | - 10.37 | 0.92 | - 8.93 |
| MS2-N44a and GW1-C36 | Spring wheat (GW1) | 7.50 | 13.69 | 40.75 | + 18.59 | 0.79 | - 21.63 |
| BN2-N18a and GW1-C36 | Spring wheat (GW1) | 8.50 | 3.708 | 40.50 | + 17.86 | 0.72 | - 28.77 |
| CG2-N11a and MS2-C77 | Spring wheat (MS2) | 4.50 | 0 | 29.20 | - 15.02 | 0.88 | - 12.5 |
| Control - residue only | Spring wheat (SN2) | ND | ND | 39.23 | | 0.90 | |
| Control - residue only | Spring wheat (MS2) | ND | ND | 23.90 | | 0.77 | |
| Control - residue only | Spring wheat(GW1) | ND | ND | 39.96 | | 1.35 | |
| | | | | | | | |
| KP2-N136b and KP1-C2 | Peas (KP1) | 26.50 | 0 | 29.10 | - 6.88 | 1.18 | + 0.089 |
| KP2-N136b and KP1-C31 | Peas (KP1) | 24.50 | 0 | 23.68 | - 24.48 | 1.17 | - 0.76 |
| BN2-N18a and KP1-C2 | Peas (KP1) | 23.00 | 2.218 | 27.30 | - 12.64 | 1.14 | - 3.31 |
| BN2-N18a and KP1-C31 | Peas (KP1) | 22.50 | 1.816 | 24.50 | - 21.60 | 1.18 | + 0.09 |
| KP2-N167a and KP1-C31 | Peas (KP1) | 20.00 | 2.333 | 21.60 | - 30.88 | 1.03 | - 12.64 |
| CG2-N11a and RT2-C36 | Peas (RT2) | 11.50 | 8.848 | 32.73 | + 4.736 | 1.20 | + 1.53 |
| KP2-N167a and KP1-C2 | Peas (KP1) | 17.50 | 1.786 | 28.10 | - 10.08 | 0.88 | - 25.28 |
| CP1-N1 and KP1-C31 | Peas (KP1) | 16.50 | 0 | 34.70 | + 11.04 | 1.02 | - 13.49 |
| YK1-N18a and RT2-C36 | Peas (RT2) | 10.00 | 0 | 33.10 | + 5.92 | 1.21 | + 2.89 |
| QA1-N61a and KP1-C31 | Peas (KP1) | 16.00 | 1.468 | 31.90 | + 2.08 | 1.27 | + 7.72 |
| QX1-N107b and KP1-C31 | Peas (KP1) | 19.00 | 2.415 | 30.40 | - 2.72 | 1.14 | - 3.31 |
| KP2-N136a and KP1-C31 | Peas (KP1) | 14.50 | 0.943 | 30.80 | - 1.44 | 1.11 | - 5.85 |
| Control - residue only | Pea residue (KP1) | ND | ND | 26.73 | | 1.13 | |
| Control - residue only | Pea residue (RT2) | ND | ND | 35.77 | | 1.23 | |

Notes: ND, not determined; Change in % C and N based on the average values of the respective control residue.